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(54) Title: BONE FORMATION-INDUCING PROTEIN		
(57) Abstract  Disclosed are a protein having a high activity for inducing bone formation. A DNA encoding the protein, a method for producing the protein and a pharmaceutical composition comprising the protein as an active ingredient.		

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improved bone formation inducing-activity.

#### Summary of the Invention

The object of the present invention is to provide a novel bone formation-inducing protein having an improved activity.

Another object of the present invention is to provide a DNA which encodes the novel bone formation-inducing protein.

Another object of the present invention is to provide a process for producing the novel bone formation-inducing protein.

Another object of the present invention is to provide a pharmaceutical composition containing the novel bone formation-inducing protein as an active ingredient.

These and other objects of the present invention will be apparent from the following description and Examples.

The above objects were achieved based on the discovery that a certain mRNA which encodes a protein having the improved bone formation inducing-activity exists in bone of vertebrates.

The first aspect of the present invention relates to a protein comprising amino acid sequence of amino acids 1 to 110 in SEQ. ID No:1 (hereinafter referred to as BIP as occasion demands) or analogous sequences thereto. The protein of amino acids 1 to 110 in SEQ. ID No:1 is one of the maturation proteins and the protein of amino acids -368 to 110 in SEQ. ID No:1 is a precursor protein thereof.

The second aspect of the present invention relates to a DNA which encodes the bone formation-inducing protein of the present invention. The DNA can be obtained by isolating a mRNA from a tissue of a vertebrate such as human and rat, constructing a cDNA library with the isolated mRNA and then screening the cDNA library using a certain probe, which is amplified by carrying out PCR using primers designed by

referring to cDNA of the prior art. to obtain clones of interest.

The third aspect of the present invention relates to a method for producing the protein. The method comprises (a) transforming a cell with the DNA and (b) culturing the transformant.

The fourth aspect of the present invention relates to a pharmaceutical composition containing the bone formation-inducing protein or active fragment thereof as an active ingredient. The bone formation-inducing protein of the present invention is useful for therapy of a disease involving a deficiency of bone such as osteoporosis, alveolar pyorrhea and the like, and bone fracture.

#### Brief Description of the Drawings

Fig.1 illustrates the technique for constructing the expression vector for the human bone formation-inducing protein of the present invention (hBIP).

Fig.2 shows the restriction map of the expression vector for the human bone formation-inducing protein of the present invention.

Fig.3 illustrates the technique for constructing the expression vector for the rat bone formation-inducing protein of the present invention (rBIP).

Fig.4 shows the restriction map of the expression vector for the rat bone formation-inducing protein of the present invention.

Figs.5 to 10 show the histological observations of the pellets 12 days after they were implanted.

Figs.11A to 11E show the autoradiograms of Northern blot analysis for poly(A)RNAs extracted from various tissues of rat.

Fig.12 illustrates the technique for making the original amino acid sequence of process site of the precursor protein of the present invention correspond to the consensus sequence.

Fig.13 illustrates the method for constructing the stable expression vector for the human bone formation-inducing protein of the present invention.

#### Detailed Description of the Invention

The present invention includes a protein comprising amino acid sequence of amino acids 1 to 110 in SEQ. ID No:1 or analogous sequences thereto. The protein of amino acids 1 to 110 in SEQ. ID No:1 is one of the maturation proteins and the protein of amino acids -368 to 110 in SEQ. ID No:1 is a precursor protein thereof.

"Analogous sequences thereto" means amino acid sequences which are substantially homologous with the amino acid sequence of amino acids 1 to 110 in SEQ. ID No:1 and constitute proteins having a bone formation-inducing activity as high as that of the protein comprising the amino acid sequence of amino acids 1 to 110 in SEQ. ID No:1. There is at least one difference between the amino acid sequence of amino acids 1 to 110 in SEQ. ID No:1 and such analogous sequences in number or kind of amino acid contained in the sequences. Examples of the difference include those caused by a replacement, an insertion or a deletion of at least one amino acid. The proteins comprising these analogous sequence are also maturation proteins. The amino acid sequence of amino acids 1 to 110 in SEQ ID No:3 may be mentioned as example of the analogous sequence to the amino acid sequence of amino acids 1 to 110 in SEQ. ID No:1. There are two differences between the two amino acid sequences in kind of amino acid contained in the sequences.

"Protein comprising amino acid sequence of amino acids 1 to 110" means a protein having a longer amino acid sequence than amino acids 1 to 110 in SEQ. ID No:1 and maintaining the bone formation inducing-

activity. For example, the following proteins are included: the precursor protein of amino acids -368 to 110 in SEQ. ID No:1, proteins analogous thereto such as a protein in which any region of amino acids -368 to -1 in SEQ. ID No:1 is replaced with another sequence, and proteins cleaved from the precursor protein of amino acids -368 to 110 at any position upstream of amino acid 1 in SEQ. ID No:1, in particular, at amino acid -1 or -2 in SEQ. ID No:1.

The protein of the present invention needs no further structural feature insofar as it has the above-mentioned amino acid sequence. Therefore, the proteins of the present invention may include any modified proteins such as a glycosylated protein, a dimerized protein, a glycosylated and dimerized protein and a mixture of these.

The present invention also includes a DNA which encodes the bone formation-inducing protein of the present invention. Examples of the DNA include DNAs comprising base sequence of nucleotides 1191 to 1520 in SEQ. ID No:1 and analogous sequences thereto. DNAs of which the base sequences are only a little analogous to the DNAs comprising base sequence of nucleotides 1191 to 1520 in SEQ. ID No:1 but are capable of encoding the bone formation-inducing protein of the present invention. Examples of the DNA sequence of nucleotides 1191 to 1520 in SEQ. ID No:1 include DNA sequence of nucleotides 87 to 1520 in SEQ. ID No:1, which is one encoding the precursor protein of amino acids -368 to 110 in SEQ. ID No:1 mentioned above.

"Analogous sequences thereto" means base sequences which are substantially homologous with the base sequence of nucleotides 1191 to 1520 in SEQ. ID No:1. There is at least one difference between the base sequence of nucleotides 1191 to 1520 in SEQ. ID No:1 and such analogous sequences in number or kind of codon contained in the sequences. Examples of the difference include those caused by a replacement, an

insertion or a deletion of at least one codon.

"DNAs of which the base sequences are only a little analogous to the DNAs comprising base sequence of nucleotides 1191 to 1520 in SEQ. ID No:1 but are capable of encoding the bone formation-inducing protein of the present invention" means those which have not few different codons from the base sequence of nucleotides 1191 to 1520 in SEQ. ID No:1 but can consequently express the bone formation-inducing protein of the present invention. Of course, it is possible to synthesize such DNA by chaining codons which each encodes an amino acid necessary to the bone formation-inducing protein of the present invention.

It is needless to say that a DNA subjected to any alteration only in coding region for polypeptide to be released from the precursor protein during processing can encode the bone formation-inducing protein of the present invention and therefore the DNA is included in the present invention.

A detailed description will now be made on the method for producing the bone formation-inducing protein and coding DNA of the present invention.

For example, the coding DNA for the bone formation-inducing protein of the present invention can be obtained as follows:

Initially, a mRNA which is a template of the cDNA of the present invention is extracted from a tissue of a vertebrate and then isolated.

The vertebrate may be a mammal. Examples of the mammal include human, rat and bovine. In particular, human and rat, such as neonatal rat, are preferred. Examples of the usable tissue include a femur, the head of a femur, a calvaria and the like. Preferred are head of femur of human and calvaria and femur of neonatal rat. However, the tissue is not limited to those mentioned here. The mRNA extraction and isolation can be carried out using a conventional technique. After the mRNA

isolation, a cDNA library is constructed using the isolated mRNA. The construction can also be carried out using a conventional technique.

On the other hand, a DNA probe is amplified for screening the target clones present in the cDNA library on the basis of oligonucleotide primers. The oligonucleotide primers can be designed by referring to the cDNA encoding the bone morphogenetic protein of the prior art.

After labeling the probe thus obtained, screening of the above-mentioned cDNA library is carried out using the labeled probe to obtain hybridized clones. When this probe is used, not only clones corresponding to the cDNA of the present invention but also clones corresponding to DNAs of the prior art hybridize. However, the clones of the present invention hybridize with the probe more weakly than those of the prior art. Therefore, the clones of the present invention can be separated from clones of the prior art by comparing the signal strength of the hybridization in the Southern method.

Once the cDNA sequence of the present invention is determined, the cDNA can be easily synthesized by referring to the sequence.

The bone formation-inducing protein of the present invention can be obtained by constructing an expression vector by integrating the above-mentioned cDNA therein, subjecting the expression vector to a transformation or transfection of a host cell, culturing the host cell, and isolating the protein secreted within the host cell or into the culture medium. It is also possible to secrete the protein in milk of a transgenic animal, such as goat or bovine, which is constructed using a DNA containing the above-mentioned cDNA of the present invention under a control of casein promoter and the like.

The expression vector usable for the present invention may be composed of one or more DNAs derived from plasmid, virus and phage into



which the cDNA of the present invention can be inserted and which is can be introduced into the host cell. The expression vector should contain a transcription promoter sequence, an enhancer sequence and/or operator sequence which control the transcription, a suitable ribosome binding site sequence, and a sequence for termination of the transcription and the translation. According to circumstances, the expression vector may contain a DNA sequence necessary for replicating itself in the host cells or a dehydrofolate reductase (dhfr) gene enabling the introduced DNA to amplify in the presence of methotrexate which is an inhibitor of dhfr. Examples of the expression vector in the case where an animal cell is used as the host cell include pcDL-SR  $\alpha$ 296 (Mol. Cell. Biol. 8, 466, 1988), pCDM8 (B. Seed, Nature 329, 840, 1987), pMAM neo (F. Lee et al., Nature 294, 228, 1981), BCMG neo (Karasuyama et al., Eur. J. Immuno., 18, 97, 1988) and pSV2 dhfr (F. Lee et al., supra). pcDL-SR  $\alpha$ 296 and pSV2 dhfr are preferable. Further, insect cells such as pAc373 (G. E. Smith et al., Proc. Natl. Acad. Sci. USA 82, 8404, 1985), yeasts such as pAM80 (Miyanojara et al., Proc. Natl. Acad. Sci. USA 80, 1, 1983) and E. coli such as pTrc99A (E. Amann et al., Gene 69, 301, 1988) can be used as the expression vector. These expression vectors may be modified if necessary.

Examples of the suitable host cells for expression of the bone formation-inducing protein of the present invention include COS-i cell, Verots cell, CHO cell, mouse C127 cell, human 293 cell, Syrian hamster BHK cell, human Namalwa cell and monkey Vero cell. Preferable host cells are COS-i cell, human 293 cell and Syrian hamster BHK cell. Although the medium used for transformation or transfection of the host cell varies depending on the host cell used, DMEM containing fetal bovine serum,  $\alpha$ MED, Ham12, RPMI-1640 and the like are usable. DMEM is preferable for COS-i cell and  $\alpha$ MED is preferable for CHO cell.

Preferable combinations of expression vector and the host cell are, for example, pcDL-SR $\alpha$ 296 vector/COS-1 cell in case of a transient expression and pSV2 dhfr vector/Syrian hamster BHK cell in case of a stable expression.

After being subjected to transformation or transfection, the host cells are allowed to culture under the condition suitable for expressing the protein. If the protein is secreted in the medium, it may be directly purified from the medium by removing the host cells. On the other hand, if the protein is accumulated within the host cells, it is isolated from cell lysates.

In the biological synthesis of the bone formation-inducing protein of the present invention, its precursor protein, which is not active, is formed in a host cell in the first place and subsequently processed to the maturation protein, which is active, at its process site with a protease. It is desirable for the amino acid sequence of this process site of the precursor protein to be efficiently cleaved with the protease in order to produce the maturation protein of the present invention to a high degree. The fact that many known precursor proteins have amino acid sequence of Arg-X-Arg/Lys-Arg (X representing an essential amino acid) as process site has been known and Nakayama et al showed that this sequence is a consensus sequence (Nakayama et al. J. Biol. Chem. 267, 16335-16345 (1992)). The precursor protein is cleaved at C-terminus of the last Arg in this sequence and this processing with the protease should be efficient.

On the other hand, it is considered that the process site of the precursor protein of the human bone formation-inducing protein of the present invention has the amino acid sequence of Ala-Arg-Arg-Lys which corresponds to amino acids -4 to -1 in SEQ. ID No:1. However, this sequence is different from the above-mentioned consensus sequence.

Therefore, it is preferable to make the original amino acid sequence correspond to the above-mentioned consensus sequence in order to produce the human bone formation-inducing protein of the present invention efficiently. For this, it is possible to employ, for example, a technique in which an original DNA sequence encoding an amino acid sequence containing the process site of the precursor protein of the present invention (corresponding to nucleotides 1179 to 1190 in SEQ. ID No:1) is replaced with a DNA sequence designed to encode an amino acid sequence containing the consensus sequence. For example, a synthesized DNA containing a base sequence which encodes the process sequence of human BMP-2 type (Arg-Glu-Lys-Arg)(J.P. KOHYO No. Hei 2-500241) or human proactivin A type (Arg-Arg-Arg-Arg)(D. Huylebroeck et al., Mol. Endocrinol. 4, 1153 (1990)) can be employed for this technique in order to make the process sequence of the human bone formation-inducing protein of the present invention correspond to the consensus sequence, since the process sequences of human BMP-2 type and human proactivin A type correspond to the consensus sequence.

When a stably-producing cell is constructed using the thus-replaced DNA and a suitable host cell, it is possible to produce the human bone formation-inducing protein of the present invention more efficiently. Examples of host cells suitable for constructing the stably-producing mutant include human 293 cell, Syrian hamster BHK cell, human Namalwa cell, monkey Vero cell and the like.

Further, the present invention includes a pharmaceutical composition containing the bone formation-inducing protein or active fragment thereof obtained in the manner as described above as an active ingredient.

The composition is useful for therapy of a disease involving osteoporosis, a bone deficiency such as alveolar pyorrhea and the like.

and bone fracture.

The pharmaceutical composition of the present invention may be administered through various routes, for example, orally or by injection or by implantation in a bone-lost site and, for this purpose, it is usually prepared in the form of, for example, a suitable formulation for oral administration, injection or implantation. Particularly, the injection may be prepared in the form of a formulation suitable to reach to the bone-lost site and, in case of the implantation, the active ingredient may be implanted in the bone-lost site with a matrix. Examples of the suitable formulations for oral administration include powder, granule, tablet, capsule, solution for internal use, emulsion or suspension.

The bone formation-inducing protein of the present invention may be administered alone or in the form of a mixture with one or more pharmaceutically-acceptable carriers suitable for the formulation concerned. These formulations can be prepared using conventional techniques for preparing a pharmaceutical composition. For example, they may be prepared by dissolving, emulsifying or suspending an ingredient or ingredients thereof in a suitable solvent.

Examples of carriers suitable for the powder, the granule, the tablet, the capsule and the like include excipients such as lactose, glucose, D-mannitol, starch, crystalline cellulose, calcium carbonate, kaolin and the like; binding agents such as starch paste solution, gelatin solution, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, polyvinylpyrrolidone, ethanol and the like; disintegrators such as starch, gelatin powder, carboxymethyl cellulose, carboxymethyl cellulose calcium salt and the like; lubricants such as magnesium stearate, talc and the like; and coating agents such as hydroxypropyl methyl cellulose, acetyl cellulose, white sugar, titanium oxide and the

like. Coloring agents, flavoring agents and the like can be optionally added to these formulations.

Examples of carriers suitable for the solution for internal use include preservatives such as benzoic acid, esters of p-hydroxy benzoic acid, dehydroacetic acid sodium salt and the like; suspending agents such as gum arabic, tragacanth, carboxymethyl cellulose sodium salt, methyl cellulose, egg yolk, surfactants and the like; edulcorants such as white sugar, syrup, citric acid and the like. Coloring agents, stabilizers and the like can be optionally added to the solution. The solvent used for the solution is mainly purified water. However, ethanol, glycerin, propylene glycol and the like are usable.

Examples of the solvents suitable for the injection include distilled water, water for injection, non-aqueous solvents such as ethanol, glycerin, propylene glycol, macrogol and the like. Examples of carriers suitable for the injection include buffers such as sodium monohydrogen phosphate, sodium dihydrogen phosphate, sodium phosphate and the like; isotonic agents such as glucose, sodium chloride and the like; preservatives such as phenol, thimerosal, esters of p-hydroxy benzoic acid and the like; stabilizers such as sodium hydrogen sulfite and the like. Painkillers, solubilizers and the like can be optionally added to the injection.

The matrix for the implantation can be either a biologically-absorbable or biologically-unabsorbable one. Examples of biologically-absorbable matrices include non-biological substances such as hydroxyapatite, poly(lactic acid), calcium sulfate and the like; and biological substances such as type I collagen, bone and the like. On the other hand, examples of biologically-unabsorbable matrices include ceramics, titanium and the like. The active ingredient can be implanted with one or more these matrices.

Generally, the injection is mainly applied to treat osteoporosis and, as described above, the implantation in a bone-lost site is applied to treat alveolar pyorrhea or fracture. However, oral administration and injection can also be applied to treat alveolar pyorrhea or bone fracture in addition to the implantation.

The dose of the active ingredient contained in the pharmaceutical composition of the present invention varies widely depending on the administration route, type of formulation, kind of disease, age and sex of the patient, and the like. However, in general, it is 0.01 to 100 mg/day for adult. When the active ingredient is implanted in the bone-lost site in the form of a mixture with collagen, the mixing ratio of the active ingredient to collagen is  $4 \times 10^{-5}$  to  $4 \times 10^{-1}$  wt%, preferably  $4 \times 10^{-5}$  to  $4 \times 10^{-2}$  wt%, more preferably  $4 \times 10^{-5}$  to  $4 \times 10^{-3}$  wt%. The amount of the mixture to be implanted can be suitably determined by the physician according to the severity of the disease.

By the present invention, prevention and complete cure of osteoporosis and the teeth deciduation caused by alveolar pyorrhea become possible and the time required for healing a fracture can be shortened.

The following examples are given to further illustrate the present invention but are not meant in any way to restrict the effective scope of the invention.

#### Example 1

Isolation of cDNA encoding the human bone formation-inducing protein

#### Preparation of probe

The following primers:

Primer 1 5'-AGCCATCAAATCATGCTACC-3'

Primer 2 5'-TCTGCAAGCGCAAGACTCTA-3'

were synthesized using an automatic DNA synthesizer and then a DNA fragment was amplified using 250nM of Primer 1 and 250nM of Primer 2 from 1  $\mu$ g:100  $\mu$ l of human placenta chromosomal DNA by the PCR method in the following solution for 25 cycles:

Solution for PCR:

10mM Tris-hydrochloride (pH 8.3), 50mM KCl, 1.5mM MgCl<sub>2</sub>, 0.001% gelatin, 200  $\mu$ M dATP, 200  $\mu$ M dCTP, 200  $\mu$ M dGTP, 200  $\mu$ M dTTP, 2.5 units AmpliTaq (Perkin Elmer Cetus)

Condition for PCR:

94°C for 1 minute, 37 °C for 2 minutes and 72 °C for 3 minutes

After the reactions, the DNAs amplified were electrophoresed on agarose gel to recover a DNA fragment having about 180bp using the glass adsorption method. This fragment was ligated with EcoRI adapter (Pharmacia) using T4 DNA ligase in the conventional manner and then the resulting fragment was ligated to the recognition site for restriction enzyme EcoRI of plasmid BlueScriptIIISK<sup>+</sup> (Stratagene). A competent cells of E.coli (JM 109) prepared by the method of Inoue et al (Inoue et al., Gene, 96, 23, 1990) were transformed with this reaction solution.

The resulting transformant was cultured in LB medium and then the plasmid DNA was isolated from lysate of the transformant by the alkali-SDS method (Birnboim et al., Nucleic Acids Res., 7, 1513, 1979). Subsequently, the plasmid DNA was subjected to removal of RNA present therein by RNaseA treatment and deproteinized by the PEG (polyethyleneglycol) precipitation method and the phenol extraction method and then reprecipitated by adding ethanol. The sequence of the plasmid DNA thus obtained was determined by the dideoxynucleotide method with T7 DNA polymerase (Sanger et al., Proc. Natl. Acad. Sci. USA., 74, 5463, 1977) after denaturing the DNA by the method of Hattori

et al (Hattori et al., Analytical Biochemistry 152, 232, 1986). The base sequence is shown in SEQ ID No:2.

Next, this plasmid DNA was digested by restriction enzyme EcoRI and the digest was electrophoresed on 1% agarose gel to recover a fragment having about 180bp using the glass adsorption method. This fragment was used as the probe.

#### Construction of cDNA library of human bone tissue

1.9mg of total RNA was isolated from 42g of head of human femur by the acid guanidine thiocyanate/phenol/chloroform extraction method (Analytical Biochemistry 162, 156, 1987) and, from 1.3mg of the RNA thus isolated, poly(A)RNA was purified using oligo-dT latex (Nippon Rocne). Double stranded DNA were synthesized from 5  $\mu$ g of the poly(A) RNA according to the method of Gubler and Hoffman. After ligating this cDNA with EcoRI adapter using T4 DNA ligase, the ligated cDNA was electrophoresed on 1% agarose gel to obtain a fraction having 2 to 5kb through an extraction. These extracted cDNA were ligated to the recognition site of restriction enzyme EcoRI of lambda phage  $\lambda$ gt10arm (Murray et al., Mol. gen. Genet., 150, 53, 1977)(Bethesda laboratory) and then an in vitro packaging (Collins et al., Proc. Natl. Acad. Sci. USA., 71, 4242, 1978) was conducted to construct the cDNA library.

#### Cloning of the cDNA

E.coli C600fhl (DNA cloning, 1, 56, 1985) was infected with recombinant phage contained in the above cDNA library and then 1,000,000 of the resulting plaques were fixed onto a nitrocellulose filter (Benton et al., Science, 196, 180, 1977). The filter was prehybridized for 20 hours at 37 °C in a hybridization solution (20% formamide, 6 $\times$ SSPE, 0.1% SDS, 100  $\mu$ g/ml salmon sperm DNA) and then was hybridized with the above-prepared probe, which was labeled with ( $\alpha$ -<sup>32</sup>P)dCTP using a multiprime-labeling kit (Amersham), at 42 °C in the



above hybridization solution. Subsequently, this filter was washed in  $2 \times$  SSC, 0.1% SDS solution at 50°C and 16 positive clones were obtained by autoradiography. The positive clones were purified in the conventional manner and DNAs thereof were prepared. These DNA fragments, which were inserted into the recombinant phage DNA, were identified to be those of interest by the method of Southern (J. Mol. Biol., 98, 503, 1975) using hybridization with the above probe. Base sequences of these DNA were determined to be identical with each other and the clones were designated as HE24, 25 and 27, respectively.

#### Determination of base sequence of the cDNA

The recombinant phage DNA thus-obtained was digested by restriction enzyme NotI and then the digest was electrophoresed on agarose gel to isolate the DNA fragment. After purification by the glass adsorption method, the purified DNA fragment was inserted into the NotI site of plasmid BlueScript[ISK+ to subclone. The E. coli containing HE24 was deposited with the Fermentation Research Institute, Agency of Industrial Science and Technology on July 3, 1992 (Acceptance No. FERM P-13045).

The partial base sequence of this cDNA was determined by the dideoxynucleotide method (Sanger et al., supra) and shown in SEQ. ID No:1 together with the amino acid sequence deduced therefrom.

From these results, it can be seen that the human bone formation-inducing protein is contained in the amino acid sequence of 478 amino acids encoded by the base sequence of nucleotides 87 to 1520 in SEQ. ID No:1. That is, the protein of the 478 amino acids was biologically synthesized in the form of a precursor protein, which includes a secretion signal, and then subjected to processing at the process site of amino acid -2, -1 or 1 in SEQ. ID No:1 to form the maturation proteins having 112, 111 or 110 amino acids, respectively.

Example 2Isolation of cDNA encoding the rat bone formation-inducing protein  
Construction of cDNA library of rat bone tissue

5.8mg of total RNA was isolated from 2g of femur of neonatal rat by the acid guanidine thiocyanate/phenol/chloroform extraction method (Analytical Biochemistry 162, 156, 1987) and poly(A)RNA was purified from 2mg of the isolated RNA using oligo-dT latex (Nippon Roche). A double stranded DNA was synthesized from 5  $\mu$ g of the poly(A)RNA according to the method of Gubler and Hoffman. After ligating this cDNA with EcoRI adapter using T4 ligase, the ligated cDNA was electrophoresed on 1% agarose gel to obtain a fraction having 1.2 to 5kb through an extraction. This extracted cDNA was ligated with the recognition site of restriction enzyme EcoRI of lambda phage  $\lambda$ gt10arm (Murray et al., Mol. gen. Genet., 150, 53, 1977) (Bethesda laboratory) and then an in vitro packaging (Collins et al., Proc. Natl. Acad. Sci. USA., 71, 4242, 1978) was conducted to construct the cDNA library.

Cloning of the cDNA

E.coli C600fhl (DNA 1, 56, 1985) was infected with recombinant phage contained in the above cDNA library and then 1,000,000 of the resulting plaques were fixed onto nitrocellulose filters (Benton et al., Science, 196, 180, 1977). The filters were prehybridized at 37°C in a hybridization solution (20% formamide, 6 $\times$ SSPE, 5 $\times$ Denhardt's, 0.5% SDS, 100  $\mu$ g/ml salmon sperm DNA) and then were hybridized with the probe obtained in Example 1, which was labeled with ( $\alpha$ - $^{32}$ P)dCTP using multiprime-labeling kit (Amersham), at 42 °C in the above hybridization solution. Subsequently, after these filters were washed with 2 $\times$ SSC, 0.1% SDS solution at 50°C and 24 positive clones were obtained through autoradiography. The positive clones were purified in the conventional

manner and DNAs thereof were prepared. These DNA fragments, which were inserted into the recombinant phage, were identified by the method of Southern hybridization (J. Mol. Biol., 98, 503, 1975) using the above probe. Base sequences of these DNA were determined to be identical with each other and the clones were designated as RB12, 45 and RC27, respectively.

#### Determination of base sequence of the cDNA

The recombinant phage DNA thus-obtained was digested by restriction enzyme NotI and then the digest was electrophoresed on agarose gel to isolate the DNA fragment. After purification by the glass adsorption method, the purified DNA fragment was inserted into the NotI site of plasmid BlueScriptII SK+ to subclone. The E. coli carrying RB45 was deposited with Fermentation Research Institute, Agency of Industrial Science and Technology on July 3, 1992 (Acceptance No. FERM P-13046).

The base sequence of this cDNA was determined by the dideoxynucleotide method (Sanger et al., supra) and shown in SEQ ID No:3 together with the amino acid sequence deduced therefrom.

From these results, it can be seen that the rat bone formation-inducing protein is composed of 476 amino acids encoded by the base sequence of nucleotides 60 to 1487 in SEQ. ID No:3. The protein of the 476 amino acids was further subjected to processing to form the maturation protein having 112, 111 or 110 amino acids, which is encoded by the base sequence of nucleotides 1152 to 1487, 1155 to 1487 or 1158 to 1487 in SEQ. ID No:3.

#### Example 3

Expression of the human bone formation-inducing protein in COS-1 cell  
To demonstrate that the human bone formation-inducing protein of

the present invention has bone formation-inducing activity. the cDNA obtained in Example 1 was ligated with a transient expression vector and the ligated vector was introduced into COS-1 cells (Gluzman: Cell. 23, 175, 1981) to express the protein. For the protein secreted in the supernatant of the medium used, the activity was measured.

#### Construction of the expression vector

pcDL-SRD obtained by modifying pcDL-SR $\alpha$ 296 reported by Takebe et al (Takebe et al., Mol. Cell. Biol., 8(1) 466, 1988) was used as the transient expression vector. Namely, this vector was obtained by digesting vector pcDL-SR  $\alpha$ 296 by restriction enzymes KpnI and PstI, blunting with DNA polymerase I and inserting EcoRI linker thereinto.

First, non-coding regions were removed from the cDNA coding for the human bone formation-inducing protein of the present invention by the method illustrated in Fig.1. In detail, HE24 was digested by restriction enzyme NcoI to remove the non-coding region present on the 5'-side thereof and, after blunting of the terminus of the digest using DNA polymerase I, the digest was further digested by restriction enzyme SalI to isolate Nco $\Delta$ -SalI fragment which contained the initiation codon and was a fragment present on the 5'-side.

On the other hand, HE24 was also digested by restriction enzymes SalI and KpnI to isolate Sal-Kpn fragment.

These two DNA fragments were ligated to the vector which was obtained by digesting BlueScriptIIISK+ by restriction enzyme HindIII, blunting the terminus thereof using DNA polymerase I and then digesting again by restriction enzyme KpnI. The resulting plasmid was digested by restriction enzymes EcoRV and KpnI to isolate EV-Kpn fragment.

Next, the following two primers for PCR:

C-1 5'-GATATCTCACCGGCAGGCACAGGTG-3'

C-2 5'-TCCCGGAGGTACCTGAAGGT-3'

were synthesized in order to amplify a cDNA region corresponding to C-terminus of the protein of the present invention.

The PCR reaction was carried out on 1  $\mu$ M of Primer C-1 and 1  $\mu$ M of Primer C-2 using HE24 as a template in the following solution for 32 cycles:

Solution for PCR

10mM Tris-hydrochloride (pH 8.3), 50mM KCl, 1.5mM  $MgCl_2$ , 0.001% gelatin, 200  $\mu$ M dATP, 200  $\mu$ M dCTP, 200  $\mu$ M dGTP, 200  $\mu$ M dTTP, 5 units AmpliTaq (Perkin Elmer Cetus), 500pg HE 24 plasmid DNA/50  $\mu$ l

Condition for PCR

92°C for 1 minute, 50 °C for 2 minutes and 72 °C for 3 minutes

After the reaction, the DNAs were electrophoresed to separate them from the reaction solution and then the DNA fragment of interest was isolated and purified using the glass adsorption method.

From the fragment thus-obtained, KpnI-EcoRV (Kpn-EV) fragment, which is DNA corresponding to the C-terminus side, was isolated and ligated to T-vector in which dTTP was attached to the 3'-terminus of the recognition site for restriction enzyme EcoRV of plasmid Blue ScriptII SK+ and then the base sequence thereof was determined in the same manner as in Example 1.

The resulting plasmid was digested by restriction enzymes KpnI and EcoRV to isolate the Kpn-EV fragment. This fragment was ligated with the above-isolated EV-Kpn fragment and BlueScriptII SK+ which was digested by restriction enzyme EcoRV and then treated with alkaline phosphatase to dephosphorize. As a result, a cDNA was obtained, in which all of the coding regions remained and no non-coding region remained.

This cDNA was isolated from the BlueScriptII SK+/EV by digesting

by restriction enzyme EcoRV and then ligated to the blunted terminus of the EcoRI recognition site of the above-mentioned pcDL-SRD vector to construct an expression vector for the human bone formation-inducing protein (SRD-hBIP). A restriction map of the plasmid thus-obtained is shown in Fig. 2.

Transfection with the expression vector for the human bone formation-inducing protein and production of the protein

According to the method of Sambrook et al (Sambrook et al., Molecular Cloning: Spring Harbor Laboratory Press, 16, 41, 1989), the vector SRD-hBIP thus-obtained was transfected into COS-1 cells to produce the human bone formation-inducing protein.

A 100mm culture dish containing medium (10ml of DMEM containing 10 v/v% fetal calf serum) was inoculated with  $1 \times 10^6$  of COS-1 cells, incubated at 37 °C for 18 hours with 5% CO<sub>2</sub> gassing and then washed with 10ml of phosphate buffered saline (PBS(-)) twice. To this, 2  $\mu$ g of mixture of SRD-hBIP DNA and DEAE dextran was added. After incubating the cells at room temperature for 15 minutes, 5ml of DMEM containing 10 v/v% fetal calf serum and 100  $\mu$ M of chloroquine was added thereto. After culturing the cells at 37°C for 3.5 hours with 5% CO<sub>2</sub> gassing, the medium was removed and then 900  $\mu$ l of 10 v/v% DMEM containing fetal calf serum and 10 v/v% dimethyl sulfoxide (DMSO) was added thereto. The cells were incubated at room temperature for 2 minutes without 5% CO<sub>2</sub> gassing, washed with 5ml of PBS(-) and 5ml of DMEM containing 10% fetal calf serum in this order and then 10ml of DMEM/10% fetal calf serum was added thereto. After incubating the cells at 37°C for 24 hours with 5% CO<sub>2</sub> gassing, the medium was removed and then 10ml of DMEM containing 2% fetal calf serum was added thereto to further incubate at 37°C with 5% CO<sub>2</sub> gassing. After 5 days, the cells were removed from the medium by centrifugation at 10,000 rpm at 4°C for 10 minutes to obtain the

conditioned medium.

To 1.400ml of the conditioned medium, urea and Tris-hydrochloride (pH 7.0) was added to yield a final concentration of 6M urea and 50mM Tris-hydrochloride (pH 7.0). The mixture was loaded to a Heparin-Sepharose column (20ml, Pharmacia) preliminarily equilibrated with 6M urea/50mM Tris-hydrochloride (pH 7.0). The column was washed with the same buffer, whereafter the elution was carried out with 30ml of the same buffer but containing 0.5M sodium chloride. The eluate was concentrated to about 5ml by ultrafiltration using a centricon, dialyzed against deionized water and freeze-dried. Subsequently, the freeze-dried proteins were dissolved in 1% sodium deoxycholate/50mM Tris-hydrochloride buffer (pH 8.0) and then the solution was loaded to a ConA-Sepharose column (200  $\mu$ l, Pharmacia) preliminarily equilibrated with 1% sodium deoxycholate/50mM Tris-hydrochloride buffer. The column was washed with the same buffer, whereafter the elution was carried out with 1ml of the same buffer but containing 0.5M methyl- $\alpha$ -D-mannoside.

As a control, SRD-hBIP vector not containing the cDNA encoding the human bone formation-inducing protein was transfected into COS-1 cells and treated in the same manner as described above.

#### Assay for the bone formation-inducing activity

The bone formation-inducing activities were measured for the above-described fractions, i.e., ConA-bound fraction and ConA-unbound fraction by the method of Sampath et al (Sampath et al., Proc. Natl. Acad. Sci. USA., 80, 6591, 1983).

First, a matrix residue was prepared from a rat-decalcified bone by removing the bone formation-inducing active ingredient with 4M guanidine hydrochloride, then a pellet was prepared by coprecipitating 25mg of the matrix residue and the protein sample contained in the ConA-bound fraction. Subsequently, this pellet was implanted subcutaneously

in the abdominothoracic part of a rat 21-28 days old and removed from the rat after 12 days. For this pellet, alkaline phosphatase activity and amount of calcium therein were measured. These procedures were repeated also on the ConA-bound fraction of the control. These results are shown in Table 1 below.

Table 1

Sample	Alkaline phosphatase activity unit/implant	Calcium mg/implant
ConA-bound fraction	27.5	9.0
ConA-unbound fraction	0.76	0.24
ConA-bound fraction (Control)	2.98	0.0

As can be seen from Table 1, the ConA-bound fraction containing the protein of the present invention exhibited very high bone formation-inducing activity in comparison with the control. It is evident from these data that the protein of the present invention has high bone formation-inducing activity.

#### Example 4

Expression of the human bone formation-inducing protein in 293T cell

To demonstrate that the human bone formation-inducing protein of the present invention expressed in 293T (J. Gen. Virology, 36, 59-72 (1977)) cell has bone formation-inducing activity, the transient expression vector constructed in Example 3 was introduced into 293T cells to produce the protein. For the protein secreted in the supernatant of the medium used, the activity was measured.

Transfection with the expression vector for the human bone formation-inducing protein and production of the protein

The transient expression vector constructed in Example 3 (SRD-



hBIP) was transfected into 293T cells to produce the human bone formation-inducing protein.

A 100ml culture dish containing medium (10ml of MEM containing 10 v/v% fetal calf serum) was inoculated with  $1 \times 10^6$  of 293T cells, incubated at 37°C for 18 hours with 5% CO<sub>2</sub> gassing and then 20 µg of a precipitate of calcium phosphate which was prepared using the SRD-hBIP constructed in Example 3 was added thereto. After incubating the cells for 18 hours with 5% CO<sub>2</sub> gassing, the medium was removed. 10ml of MEM containing 2 v/v% fetal calf serum was added thereto and then the cells was incubated at 37 °C with 5% CO<sub>2</sub> gassing. After 3 days, the cells were removed from the medium by centrifugation at 10,000 rpm at 4°C for 10 minutes to obtain the conditioned medium.

To 1,400ml of the conditioned medium, urea and Tris-hydrochloride (pH 7.0) was added to yield a final concentration of 6M urea and 50mM Tris-hydrochloride (pH 7.0). The mixture was loaded to a Heparin-Sepharose column (20ml, Pharmacia) preliminarily equilibrated with 6M urea/50mM Tris-hydrochloride (pH 7.0). The column was washed with the same buffer, whereafter the elution was carried out with 30ml of the same buffer but containing 0.5M sodium chloride. The eluate was concentrated to about 5ml by ultrafiltration using a centricon, dialyzed against deionized water and then freeze-dried. Subsequently, the freeze-dried proteins were dissolved in 1% sodium deoxycholate/50mM Tris-hydrochloride buffer (pH 8.0) and then the solution was loaded to a ConA-Sepharose column (200 µl, Pharmacia) preliminarily equilibrated with 1% sodium deoxycholate/50mM Tris-hydrochloride buffer. The column was washed with the same buffer, whereafter the elution was carried out with 1ml of the same buffer but containing 0.5M methyl- $\alpha$ -D-mannoside.

As a control, pcDL-SRD vector not containing the cDNA encoding the human bone formation-inducing protein was transfected into 293T

cells and treated in the same manner as described above.

#### Assay for the bone formation-inducing activity

The bone formation-inducing activities were measured in the same manner as Example 3.

Namely, a pellet was prepared by coprecipitating 25mg of the matrix residue and the protein sample obtained above, then implanted subcutaneously in the abdominothoracic part of a rat 21-28 days old. After 12 days, the pellet was removed from the rat and alkaline phosphatase activity and amount of calcium therein were measured. These procedures were repeated also on the ConA-bound fraction of the control. The results are shown in Table 2 below.

Table 2

Sample	Alkaline phosphatase activity unit/implant	Calcium mg/implant
ConA-bound fraction	10.3	0.94
ConA-unbound fraction	0.4	0.04
ConA-bound fraction (Control)	2.24	0.25
ConA-unbound fraction (Control)	0.33	0.0

As can be seen from Table 2, the ConA-bound fraction containing the protein of the present invention exhibited very high bone formation-inducing activity in comparison with the control. It is evident from this data that the protein of the present invention expressed by 293T cells also has a high bone formation-inducing activity.

#### Example 5

Expression of the rat bone formation-inducing protein in COS-1 cell

To demonstrate that the rat bone formation-inducing protein of

the present invention has bone formation-inducing activity. the cDNA obtained in Example 2 was ligated to a transient expression vector and the ligated vector was introduced into COS-1 cells to produce the protein. For the protein secreted in the conditioned medium, the activity was measured.

#### Construction of the expression vector

Similarly to in Example 3, pcDL-SRD obtained by modifying pcDL-SR  $\alpha$ 296 reported by Takebe et al was used as the transient expression vector.

Namely, this vector was obtained by digesting vector pcDL-SR  $\alpha$ 296 by restriction enzymes KpnI and PstI, blunting with DNA polymerase I and inserting EcoRI linker thereinto.

Initially, this vector was digested by restriction enzyme EcoRI, blunted using DNA polymerase I and then treated with alkaline phosphatase to dephosphorize.

On the other hand, non-coding regions of the cDNA coding for the bone formation-inducing protein of the present invention were removed by the method illustrated in Fig.3.

First, the following two sets of primers for PCR, which correspond to the N-terminus (ATG: initiation codon) and the C-terminus (TAA: stop codon) of the protein of the present invention respectively, were synthesized:

#### N-terminus primer

N-1 5' -GATATCATGGCTCCAGGTCTTGC-3'

N-2 5' -GCACGAAGCTTCGGACG-3'

#### C-terminus primer

C-1 5' -GATATCTTACCGACAGGCACAGGT-3'

C-2 5' -CCAGGAGGTACCTGAAGG-3'

The PCR reactions were carried out using 1  $\mu$ M of Primer N-1 and

1  $\mu$ M of Primer N-2 using RB45 as a template in the following solution for 30 cycles to amplify 5'-terminus DNA fragment:

Solution for PCR:

10mM Tris-hydrochloride (pH 8.3), 50mM KCl, 1.5mM  $MgCl_2$ , 0.001% gelatin, 200  $\mu$ M dATP, 200  $\mu$ M dCTP, 200  $\mu$ M dGTP, 200  $\mu$ M dTTP, 5 units AmpliTaq (Perkin Elmer Cetus), 500pg RBplasmid DNA/50  $\mu$ l

Condition for PCR:

94 °C for 1 minute, 45 °C for 2 minutes and 72 °C for 2 minutes

After the reactions, the DNAs were electrophoresed on agarose gel to separate them from the reaction solution and then DNA fragment of interest was isolated and purified using the glass adsorption method.

On the other hand, 3'-terminus DNA fragment was amplified using 1  $\mu$ M of Primer C-1 and 1  $\mu$ M of Primer C-2 under the same reaction condition as the 5'-terminus DNA fragment, and the DNA fragment was isolated and purified in the same manner as the 5'-terminus DNA fragment.

The thus-obtained EcoRV-HindIII (EV-H3) fragment, which is DNA on the 5'-terminus side, and KpnI-EcoRV (Kpn-EV) fragment, which is DNA on the 3'-terminus side, were individually ligated to T-vector described in Example 2 and then the base sequence thereof was determined in the same manner as Example 1.

The plasmid DNA ligated with the EV-H3 fragment, which is DNA on the 5'-terminus side, was digested by restriction enzymes EcoRI and HindIII to isolate the EV-H3 fragment, then the isolated EV-H3 fragment was inserted into BlueScriptSKII+ plasmid between restriction enzyme recognition sites EcoRV and KpnI together with HindIII-KpnI (H3-Kpn) fragment isolated by digesting RB45 with restriction enzymes HindIII and KpnI. Further, the plasmid DNA thus-obtained was digested by restriction enzymes EcoRV and KpnI to isolate EV-Kpn fragment.

On the other hand, the plasmid DNA ligated with the Kpn-EV

fragment was digested by restriction enzymes KpnI and EcoRV to isolate Kpn-EV fragment.

Both fragments thus-obtained were ligated to the restriction enzyme EcoRI recognition site of the above-mentioned pcDL-SRD vector, the terminus of which had been blunted, to construct an expression vector for the bone formation-inducing protein. A restriction map of the plasmid thus-obtained is shown in Fig. 4.

Transfection with the expression vector for the rat bone formation-inducing protein and production of the protein

The vector thus-obtained was transfected into COS-1 cells to produce the rat bone formation-inducing protein.

A 100mm culture dish containing medium (10ml of DMEM containing 10 v/v% fetal calf serum) was inoculated with  $1 \times 10^6$  of COS-1 cells, incubated at 37 °C for 18 hours with 5% CO<sub>2</sub> gassing and then washed with 10ml of phosphate buffered saline (PBS(-)) twice. To this, 2  $\mu$ g of mixture of the above-mentioned vector and DEAE dextran was added. After incubating the cells at room temperature for 15 minutes, 5ml of DMEM containing 10 v/v% fetal calf serum and 100  $\mu$ M of chloroquine was added thereto. After culturing the cells at 37°C for 3.5 hours with 5% CO<sub>2</sub> gassing, the medium was removed and then 900  $\mu$ l of 10 v/v% DMEM containing fetal calf serum and 10% dimethyl sulfoxide (DMSO) was added thereto. The cells were incubated at room temperature for 2 minutes without CO<sub>2</sub> gassing, washed with 5ml of PBS(-) and 5ml of DMEM containing 10 v/v% fetal calf serum in this order and then 10ml of DMEM containing 10 v/v% fetal calf serum was added thereto. After incubating the cells at 37°C for 24 hours with 5% CO<sub>2</sub> gassing, the medium was removed and then 10ml of DMEM containing 2% fetal calf serum was added thereto to further incubate at 37°C with 5% CO<sub>2</sub> gassing. After 5 days, the cells were removed from the medium by centrifugation at 10,000 rpm

at 4°C for 10 minutes to obtain the conditioned medium.

To 700ml of the conditioned medium, urea and Tris-hydrochloride (pH 7.0) was added to yield a final concentration of 6M urea and 50mM Tris-hydrochloride (pH 7.0). The mixture was loaded to a Heparin-Sepharose column (20ml, Pharmacia) preliminarily equilibrated with 6M urea/50mM Tris-hydrochloride (pH 7.0). The column was washed with the same buffer, whereafter the elution was carried out with 30ml of the same buffer but containing 0.5M sodium chloride. The eluate was concentrated to about 5ml by ultrafiltration using a centricon, dialyzed against deionized water, freeze-dried and then dissolved in 1% sodium deoxycholate/50mM Tris-hydrochloride buffer (pH 8.0). Subsequently, this solution was loaded to a ConA-Sepharose column (200  $\mu$ l, Pharmacia) preliminarily equilibrated with said buffer. The column was washed with the same buffer, whereafter the elution was carried out with 1ml of the same buffer but containing 0.5M methyl- $\alpha$ -D-mannoside.

As a control, pcDL-SRD vector not containing the cDNA encoding the human bone formation-inducing protein was transfected into COS-1 cells and treated in the same manner as described above.

#### Assay for the bone formation-inducing activity

The bone formation-inducing activities were measured for the above-described fractions, i.e., ConA-bound fraction and ConA-unbound fraction in the same manner as Example 3.

First, a pellet was prepared by coprecipitating 25mg of the matrix residue, which was prepared from a rat-decalcified bone by removing the bone formation-inducing active ingredient with 4M guanidine hydrochloride, and the obtained protein. The pellet was then implanted subcutaneously in the abdominothoracic part of a rat 21-28 days old. After 12 days, the pellet was removed from the rat and the alkaline phosphatase activity and amount of calcium therein were measured.

These procedures were repeated also on the control. These results are shown in Table 3 below.

Table 3

Sample	Alkaline phosphatase activity unit/implant	Calcium mg/implant
ConA-bound fraction	23.9	8.9
ConA-unbound fraction	0.81	0.06
Control	1.25	0.13

As can be seen from Table 3, the ConA-bound fraction containing the protein of the present invention exhibited very high bone formation-inducing activity in comparison with the control. It is evident from these data that the protein of the present invention has high bone formation-inducing activity.

Figs.5 to 10 show the histological observations of the pellets 12 days after they were implanted. Figs.5 to 7 are for the pellet prepared from the rat bone formation-inducing protein contained in the ConA-adsorbed fraction, which was partially purified, and the matrix residue of rat bone. Figs.8 to 10 are for the pellet prepared from the protein contained in the supernatant of the culture medium for the control, which was partially purified with Heparin-Sepharose, and the matrix residue of rat bone. Figs.5 and 8 are microphotographs ( $\times 150$ ) of slices of the pellet stained with hematoxylin and eosin. Figs.6 and 9 are microphotographs ( $\times 200$ ) of slices stained with PAS and alcian blue, and Figs.7 and 10 are microphotographs ( $\times 200$ ) of slices stained with von Kossa.

Fig.5 shows that the interstitial cells markedly proliferated in the area among the implanted collagen matrix particles. In this area, osteoclasts and fibroblasts can be also observed and, around the matrix

particles. cells which are considered to be osteoblasts can be also observed. Further, a proliferation of cartilage matrix containing chondrocyte can be observed. On the other hand, in the collagen matrix, it can be observed that a marked calcification occurred. The calcification can be observed in the cartilage matrix also.

In Fig.6. the cartilage cells stained with alcian blue are observed. Although the cells were weakly stained, there are strongly-stained cells scattered among the weakly-stained ones. Also, calcified substance stained with PAS can be observed.

From Fig.7. it is observed that the calcified substance is present in the collagen matrix and the cartilage matrix.

On the other hand, Figs.8 to 10 show that the cells scarcely proliferated in the area among the implanted collagen matrix particles.

It is considered that the cells present in this area are only fibroblasts. No cartilage matrix, osteoclast, osteoblast or calcified substance is observed.

Thus, it is also evident from the above figures that the protein of the present invention has an ability of inducing the bone formation.

#### Example 6

Expression distribution of mRNA of the rat bone formation-inducing protein

Poly(A)RNAs were extracted from various tissues of rat and the expression distribution thereof for mRNA of the rat bone formation-inducing protein was determined by the Northern hybridization method.

First, RNAs were individually extracted cerebellum, costa, costal cartilage, trachea, blood vessel, spleen, thymus, muscle and bone marrow of SD rats 10-15 weeks old from and femur and calvaria of neonatal SD rats by the acid guanidine thiocyanate/phenol/chloroform



extraction method and poly(A)RNAs were extracted therefrom respectively with oligo-dT latex.

10  $\mu$ g of every poly(A)RNAs was denatured in 1M glyoxal/50% dimethylsulfoxide/10mM sodium phosphate buffer (pH 7.0) at 50°C for 1 hour, then subjected to 1% agarose gel (10mM sodium phosphate buffer (pH 7.0)) electrophoresis. RNAs of known molecular weights (GIBCO BRL) and treated in the same manner as described above were used as markers.

Next, the poly(A)RNAs thus-isolated were transferred from the agarose gel to a nylon membrane (High bond N, Amersham). The filter was prehybridized at 42°C for 3 hours in a hybridization solution (40% formamide, 6 $\times$ SSPE, 5  $\times$ Denhardt's, 0.1% SDS, 100  $\mu$ g/ml salmon sperm DNA) and then was hybridized with a rat bone formation-inducing protein probe, which was labeled with ( $\alpha$ - $^{32}$ P)dCTP by a multiprime-labeling kit, at 42 °C for 20 hours in the above hybridization solution. The rat bone formation-inducing protein probe was a DNA fragment having a total length of 2.2 kb of cDNA for the rat bone formation-inducing protein which was produced by digestion of plasmid RB12 with restriction enzyme NotI. Subsequently, after washing this filter in 0.1 $\times$ SSC, 0.1% SDS solution at 50°C, an autoradiography of Northern blot analysis was conducted to determine the expression distribution and amount of mRNA of the rat bone formation-inducing protein for every tissue on the basis of signal intensity.

In carrying out this autoradiography, hybridizations were carried out also for BMP-2 and BMP-3 using rat BMP-2 cDNA and rat BMP-3 cDNA as probes to determine the expression distribution and amount thereof. BMP-2 and BMP-3 are bone morphogenetic proteins described in J.P. KOHYO Nos. Hei 2-500241 and Hei 3-503649.

Preparation of probe for rat BMP-2

DNA probe (5'-ACTAATCATGCCATTGTTTCAGACGTTGGTCAACTCTGTTAAC-3') was synthesized based on the base sequence of the human BMP-2 cDNA described in J.P. KOHYO No. Hei 2-500241. The human BMP-2 cDNA was cloned from a human placenta cDNA library using this probe. Subsequently, using a cDNA fragment containing the whole coding region for human BMP-2 as a probe, rat BMP-2 cDNA was cloned from a rat placenta cDNA library. The thus-obtained cDNA fragment containing the whole coding region for rat BMP-2 was used as a probe for the Northern hybridization.

Preparation of probe for rat BMP-3

Primer DNA was synthesized based on the base sequence of the human BMP-3 cDNA described in J.P. KOHYO No. Hei 2-500241. Using this primer, a DNA fragment containing 180bp of maturation region was amplified from the human placenta chromosome DNA by the PCR method according to the method described in Examples 1 and 2 and then subcloned into plasmid BlueScript11SK+. Using this DNA fragment as a probe, rat BMP-3 cDNA was cloned from a rat femur cDNA library. The thus-obtained cDNA fragment containing whole rat BMP-3 cDNA was used as a probe for the Northern hybridization.

Figs. 11A to 11E show the autoradiograms thus obtained. Figs. 11A and 11B are for the bone formation-inducing protein of the present invention (BIP). Fig. 11C is for BMP-2 and Figs. 11D and 11E are for BMP-3. Lanes A to K are the autoradiograms for the following tissues:

A	cerebellum	mature SD rat
B	costa	ditto
C	costal cartilage	ditto
D	trachea	ditto
E	blood vessel	ditto

F	spleen	ditto
G	thymus	ditto
H	muscle	ditto
I	bone marrow	ditto
J	femur	neonatal SD rat
K	calvaria	ditto

It can be seen from Figs. 11A and 11B that the mRNAs for the rat bone formation-inducing protein of the present invention are 4.1kb and 2.8kb in size and distributed to femur (lane J) and calvaria (lane K) of the neonatal SD rats and cerebellum (lane A), costal cartilage (lane C), trachea (lane D) and blood vessel (lane E) of the matured rat in high density.

On the other hand, Fig. 11C shows that rat BMP-2 mRNAs are 3.8kb and 2.5kb and Figs. 11D and 11E show that rat BMP-3 mRNAs are 6.7kb, 4.7kb and 2.6kb in size. Thus, the mRNAs for the rat bone formation-inducing protein of the present invention are different from those of rat BMP-2 and -3 in size. This means that the gene of the bone formation-inducing protein of the present invention is different from that of either BMP-2 or -3.

The expression distribution data also indicate that there is a great difference between the rat BMPs and the rat bone formation-inducing protein of the present invention. That is, the rat BMP-2 mRNAs are distributed to trachea (lane D) in high density but to cerebellum (lane A), costal cartilage (lane C) and blood vessel (lane E) in low density, and also the rat BMP-3 mRNAs are distributed to trachea (lane D) in high density but to cerebellum (lane A) and costal cartilage (lane C) in low level.

Thus, the rat bone formation-inducing protein of the present invention is distinctly different from BMP-2 and -3. Because of the

fact that the mRNAs for the bone formation-inducing protein of the present invention distribute to cerebellum and costal cartilage in high density, it can be expected that the protein would play an important part in the nervous system and chondrogenesis.

#### Example 7

Replacement of the process site of the human bone formation-inducing protein with the consensus sequence

In order to cleave the maturation protein from the precursor protein of the present invention efficiently, the following original DNA sequence encoding the amino acid sequence containing the process sequence of the precursor protein:

```

5' -TG CAG AAA GCC CGG AGG AAG CAG TGG GAT GAG CCG AGG GTG TGC TCC
      3' -C TTT CGG GCC TCC TTC GTC ACC CTA CTC GGC TCC CAC ACG AGG
      Glu Lys Ala Arg Arg Lys Gln Trp Asp Gln Pro Arg Val Cys Ser
      CGG AGG TAC-3'
      GCC TC-5'
      Arg Arg Tyr

```

which is shown in SEQ. ID No:1 as nucleotides 1171 to 1226 will be replaced with the following synthesized DNA containing the consensus sequence as described above:

(Human BMP-2 type)

```

5' -TG CAC AAA CGC GAG AAG AGG CAG TGG GAT GAG CCG AGG GTG TGC TCC
      3' -G TTT GCG CTC TTC TCC GTC ACC CTA CTC GGC TCC CAC ACG AGG
      His Lys Arg Gln Lys Arg
      CGG AGG TAC-3'
      GCC TC-5'

```

First, as shown in Fig.12, SK+/H3  $\Delta$ .Kpn will be digested by restriction enzymes BbsI and KpnI and then ligated with the synthesized

DNA to obtain the modified SK+/H3 $\Delta$ .Kpn. Subsequently, the modified SK+/H3 $\Delta$ .Kpn will be digested by restriction enzymes EcoRV and KpnI to obtain the EcoRV-KpnI fragment containing the precursor region and the modified portion.

On the other hand, SK+/EV as shown in Fig.12 will be digested by restriction enzymes KpnI and EcoRV to obtain the KpnI-EcoRV fragment containing the C-terminus portion of the maturation region of the human bone formation-inducing protein of the present invention.

These two DNA fragments will be inserted into the restriction enzyme EcoRV recognition site of BlueScriptII SK+. Through these procedures, the base sequence corresponding to the process site of the human bone formation-inducing protein of the present invention, which is contained in EcoRV fragment in the plasmid DNA thus obtained, will be replaced with that corresponding to the consensus sequence.

Also, the following synthesized DNA will be used for this technique:

(Human proactivin A type)

5' -TG CAG AAA CGC CGG AGG AGG CAG TGG GAT GAG CCG AGG GTG TGC TCC

3' -C TTT GCG GCC TCC TCC GTC ACC CTA CTC GGC TCC CAC ACG AGG

Gln Lys Arg Arg Arg Arg

CGG AGG TAC-3'

GCC TC-5'

Fig.12 illustrates the above procedures.

#### Example 8

Establishment of the cell producing the human bone formation-inducing protein

A stably-producing cell was established using human 293 cell as host cell.

### Construction of stable expression vector

Restriction enzyme EcoRV recognition site of vector pEVMT was digested by restriction enzyme EcoRV. whereafter the 5' terminus thereof was dephosphorized with bacterial alkaline phosphatase. To this DNA, a DNA fragment isolated by digesting the plasmid BlueScriptII SK+/EV, which was prepared in Example 3, by restriction enzyme EcoRV was ligated using T4 DNA ligase to construct a stable expression vector pEVMT(hBIP).

The same procedures were repeated using vector pEVCMV to construct a stable expression vector pEVCMV(hBIP).

### Transformation of human 293 cell and isolation of stably-producing cell

Calcium phosphate coprecipitates with 20  $\mu$ g of the stable expression vector pEVMT(hBIP) DNA and 2  $\mu$ g of pSV2neo were prepared according to the method of Chen et al (C. Chen & H. Okayama, Mol. Cell Biol., 7, 12745-12752 (1987)). These coprecipitate were added dropwise to a 100mm culture dish which had been inoculated with  $2 \times 10^5$  of human 293 cells per 1ml of medium (90% MEM and 10% fetal calf serum) and incubated at 37 °C for one evening with 5% CO<sub>2</sub> gassing. After keeping this temperature for one evening, the cells were ripped off from the dish using 0.025% trypsin/0.01% EDTA and then diluted 1:10 with a selection medium (90% MEM + 10% fetal calf serum + 2mg of G418). The diluted cells were inoculated in ten 100mm dishes, and then incubated in the same medium while freshening the medium until visible colonies were formed.

Each colony thus formed was subjected to a magnification culture in a medium and then measured for amount of the human bone formation-inducing protein secreted in the conditioned medium by an enzyme immunoassay method (EIA) to isolate a producing cells for the protein in large quantities.

The same procedures were repeated using the stable expression

vector pEVCMV(hBIP) to isolate a producing cells for the protein in large quantities.

Fig.13 illustrates the above procedures.

If the resulting cells are gradually domesticated to a high concentration of methotrexate (MTX) in the medium, a highly-expressing clones will be isolated.

## SEQUENCE LISTING

SEQ. ID NO: 1

SEQUENCE LENGTH: 1560 nucleotides

SEQUENCE TYPE: nucleotide with corresponding protein

STRANDEDNESS: double

TOPOLOGY: linear

MOLECULE TYPE: cDNA to mRNA

ORGANISM: human

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-365 -360	
CCG GGA CCC GGG CCC CAG CTG CTG CTG CTG CTG CCG TTG TTT CTG	161
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-355 -350 -345	
CTG TTG CTC CGG GAT GTG GCC GGC AGC CAC AGG GCC CCC GCC TGG TCC	209
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-340 -335 -330	
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-325 -320 -315	
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Arg His Pro Gly Asp Ala Ala Ala Thr Leu Gly Pro Ser Ala Gln Asp	
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-295 -290 -285 -280	



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Gly Ala Arg Pro Gly Gly Gly Asn Thr Val Arg Ser Phe Arg Ala Arg	
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GAG CCG CCT CCG TGG CCT CGA GCG CTC GAG GTG CTA TGC AAG CCG CCG	545
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-230 -225 -220	
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-180 -175 -170	
CAG GCC AAG GAC ATC TCC CCC ATC GTC AAG GCG GCC CGC CGG GAT GGC	737
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-150 -145 -140	

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-115                      -110                      -105	
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Tyr Asp Pro Phe Pro Ala Gly Asp Pro Glu Pro Arg Ala Ala Pro Asn	
-100                      -95                      -90	
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Asn Ser Ala Asp Pro Arg Val Arg Arg Ala Ala Gln Ala Thr Gly Pro	
-85                      -80                      -75	
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His Ala Gln His Phe His Lys His Gln Leu Trp Pro Ser Pro Phe Arg	
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Ala Leu Lys Pro Arg Pro Gly Arg Lys Asp Arg Arg Lys Lys Gly Gln	
-35                      -30                      -25	
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Glu Val Phe Met Ala Ala Ser Gln Val Leu Asp Phe Asp Glu Lys Thr	
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CGG AGG TAC CTG AAG GTG GAC TTC GCA GAC ATC GGC TGG AAT GAA TGG	1265
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10 15 20 25	
ATA ATC TCA CCG AAA TCT TTT GAT GCC TAC TAC TGC GCG GGA GCA TGT	1313
Ile Ile Ser Pro Lys Ser Phe Asp Ala Tyr Tyr Cys Ala Gly Ala Cys	
30 35 40	
GAG TTC CCC ATG CCT AAG ATC GTT CGT CCA TCC AAC CAT GCC ACC ATC	1361
Glu Phe Pro Met Pro Lys Ile Val Arg Pro Ser Asn His Ala Thr Ile	
45 50 55	
CAG AGC ATT GTC AGG GCT GTG GGC ATC ATC CCT GGC ATC CCA GAG CCC	1409
Gln Ser Ile Val Arg Ala Val Gly Ile Ile Pro Gly Ile Pro Glu Pro	
60 65 70	
TGC TGT GTT CCC GAT AAG ATG AAC TCC CTT GGG GTC CTC TTC CTG GAT	1457
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75 80 85	
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Glu Asn Arg Asn Val Val Leu Lys Val Tyr Pro Asn Met Ser Val Asp	
90 95 100 105	
ACC TGT GCC TGC CGG TGAGACCACT CCAGGGTGGG AAGAAGCCAC GCCCAGCAGA	1560
Thr Cys Ala Cys Arg	
110	

SEQ. ID NO: 2

SEQUENCE LENGTH: 182 nucleotides

SEQUENCE TYPE: nucleotide

STRANDEDNESS: double

TOPOLOGY: linear

MOLECULE TYPE: Other nucleic acid

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TTCCTGAGCC TTGCTGTGTA CCAGAAAAGA TGTCCTCACT CAGTATTTTA TTCTTTGATG    120
AAAATAAGAA TGTAGTGCTT AAAGTATACC CTAACATGAC AGTAGAGTCT TGCGCTTGCA    180
GA                                                                    182

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SEQ. ID NO: 3

SEQUENCE LENGTH: 1497 nucleotides

SEQUENCE TYPE: nucleotide with corresponding protein

STRANDEDNESS: double

TOPOLOGY: linear

MOLECULE TYPE: cDNA to mRNA

ORGANISM: rat

```

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Met Ala Pro Gly Leu Ala Arg Ile Ser Leu Arg Ser Gln Leu Leu Pro
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Arg Lys Lys Lys Asp Gln Asp Thr Phe Thr Pro Ser Ser Ser Gln Val	
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85 90 95	

TAC CCC AAT ATG TCC GTA GAG ACC TGT GCC TGT CGG TAAGGTGGCT 1497  
Tyr Pro Asn Met Ser Val Glu Thr Cys Ala Cys Arg  
100 105 110  
TCAAGATGGA AGGCAGACCT CCTTCACCCC TGCTGTGCAG AGTGGCATTG TTGGAGCCAG 1557



Claims

1. A protein comprising an amino acid sequence of amino acids 1 to 110 in SEQ. ID No:1 or analogous sequences thereto.
2. The protein according to claim 1. wherein the protein is derived from human.
3. The protein according to claim 1. wherein the protein is derived from rat.
4. A DNA encoding a protein comprising an amino acid sequence of amino acids 1 to 110 in SEQ. ID No:1 or analogous sequences thereto.
5. The DNA according to claim 4. wherein the protein is derived from human.
6. The DNA according to claim 4. wherein the protein is derived from rat.
7. The DNA according to claim 4. wherein the DNA comprises base sequence of nucleotides 1191 to 1520 in SEQ. ID No:1 or analogous sequences thereto.
8. The DNA according to claim 7. wherein the DNA comprises base sequence of nucleotides 87 to 1520 in SEQ. ID No:1 or analogous sequences thereto.
9. A method for producing a protein comprising an amino acid sequence of amino acids 1 to 110 in SEQ. ID No:1 or analogous sequences thereto. which comprises:
  - (a) transforming a cell with a DNA encoding said protein which further comprises expression-controlling sequences; and
  - (b) culturing said transformant.
10. A pharmaceutical composition comprising a protein which comprises an amino acid sequence of amino acids 1 to 110 in SEQ. ID No:1 or analogous sequences thereto or active fragment thereof as a active

ingredient together with pharmaceutically-acceptable carriers.

11. The pharmaceutical composition according to claim 10, wherein the composition is for implantation.

Fig. 1

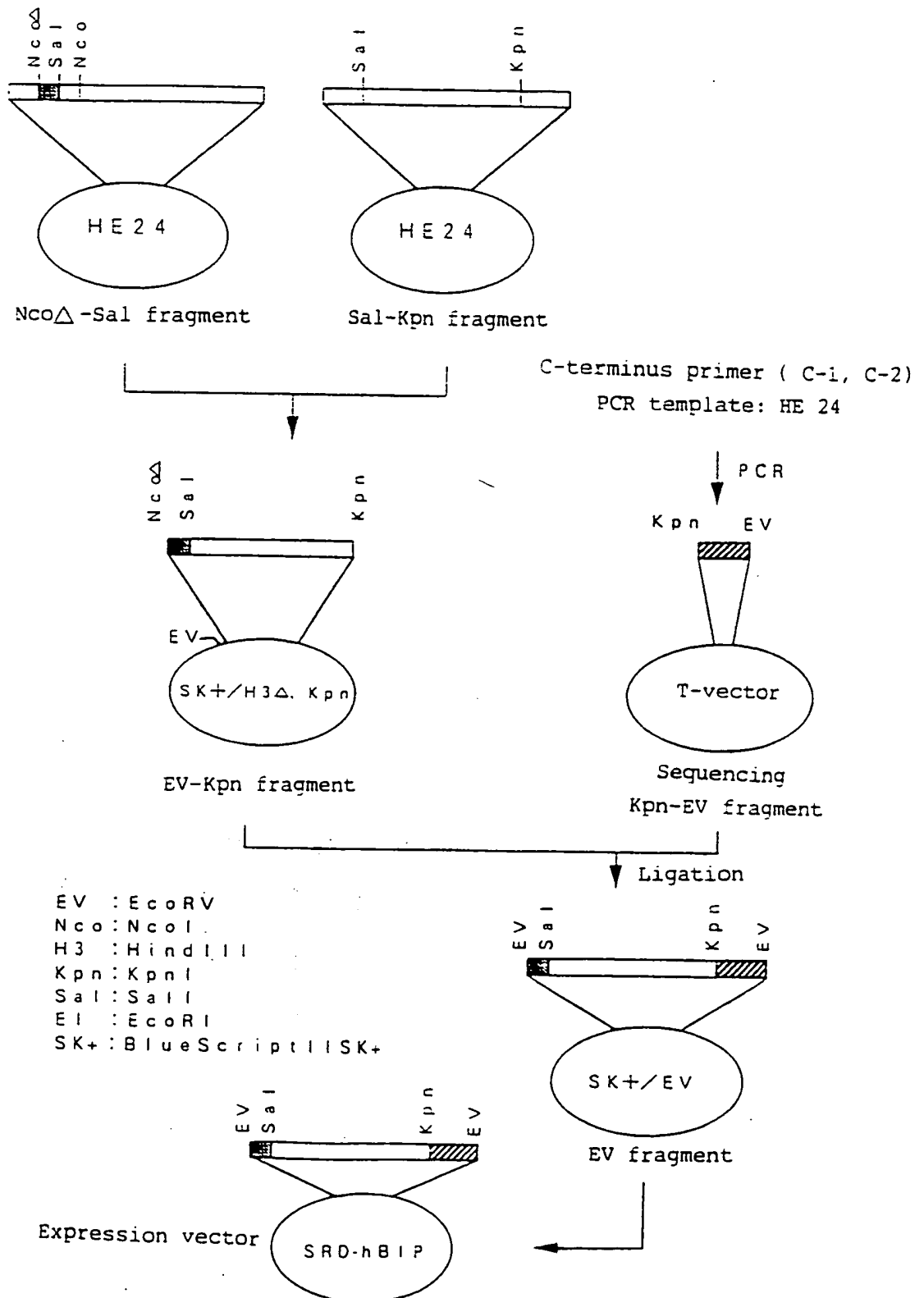


Fig. 2

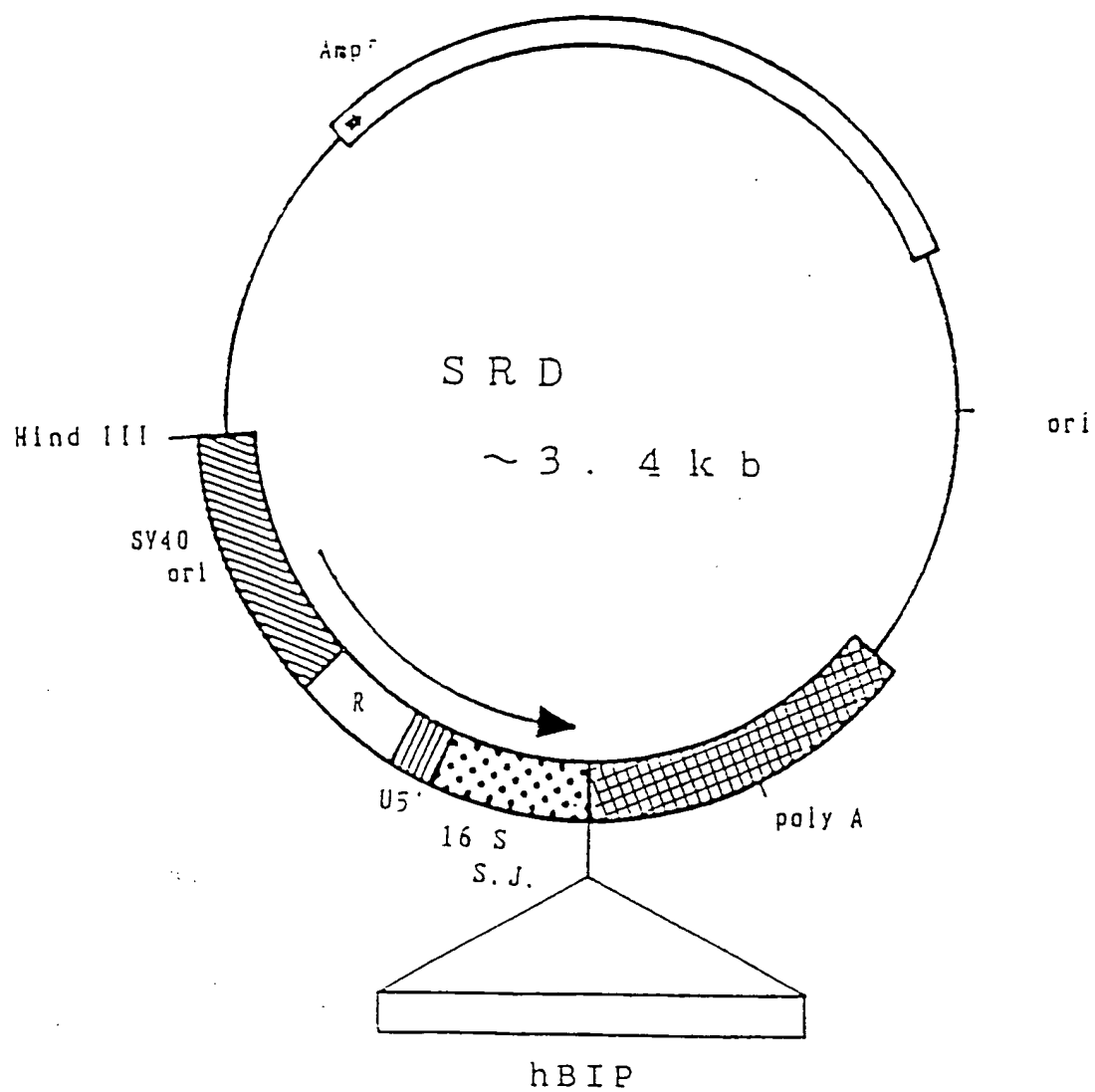


Fig. 3

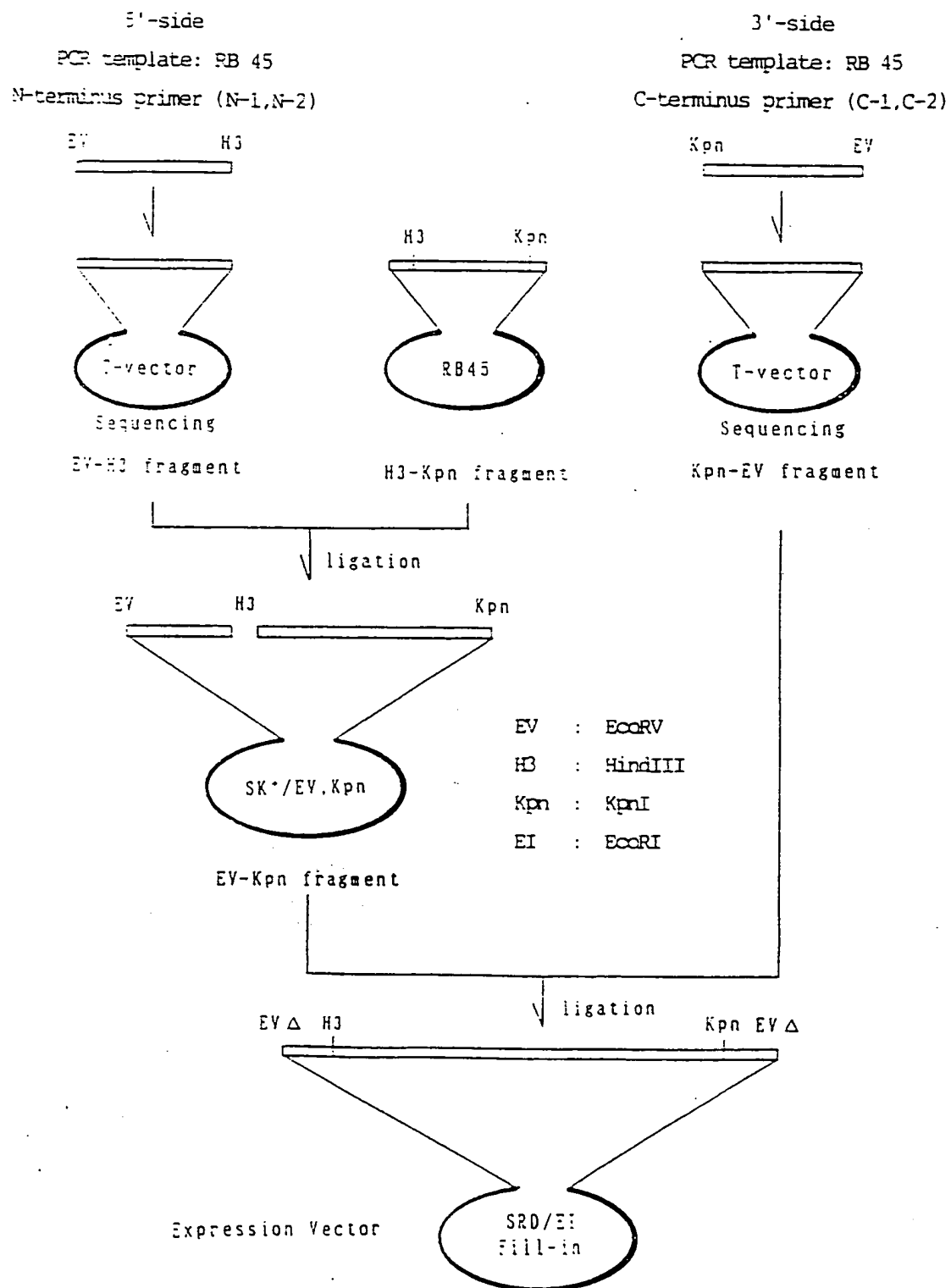


Fig. 4

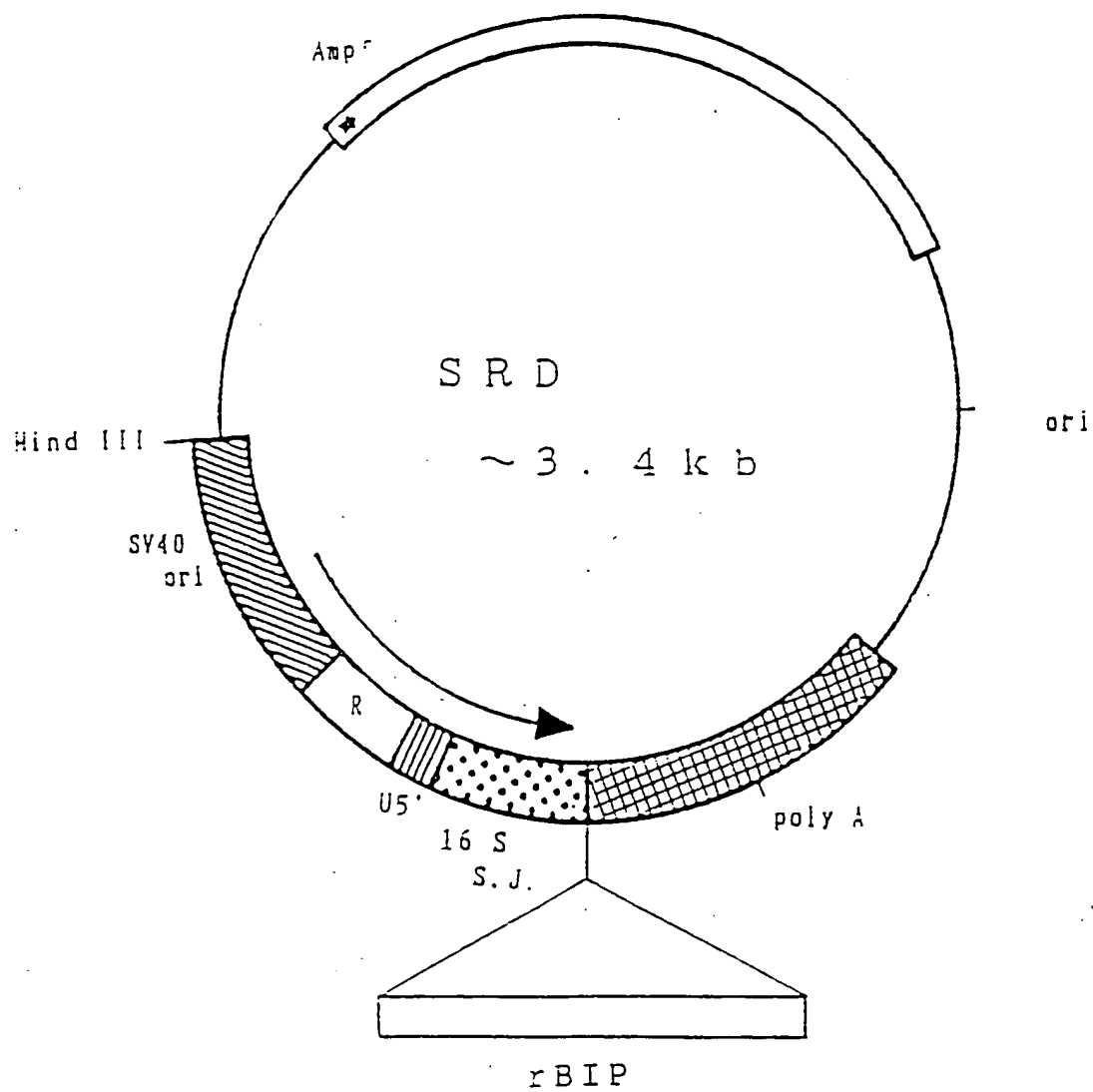


Fig. 5

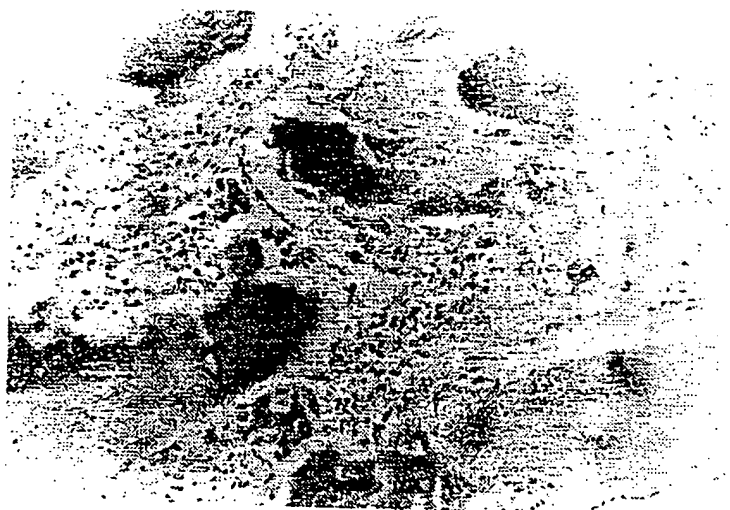


Fig. 6





Fig. 7

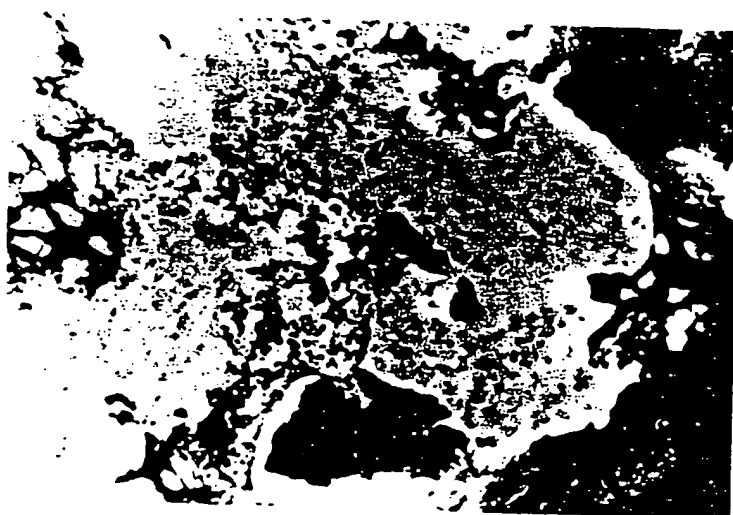


Fig. 3

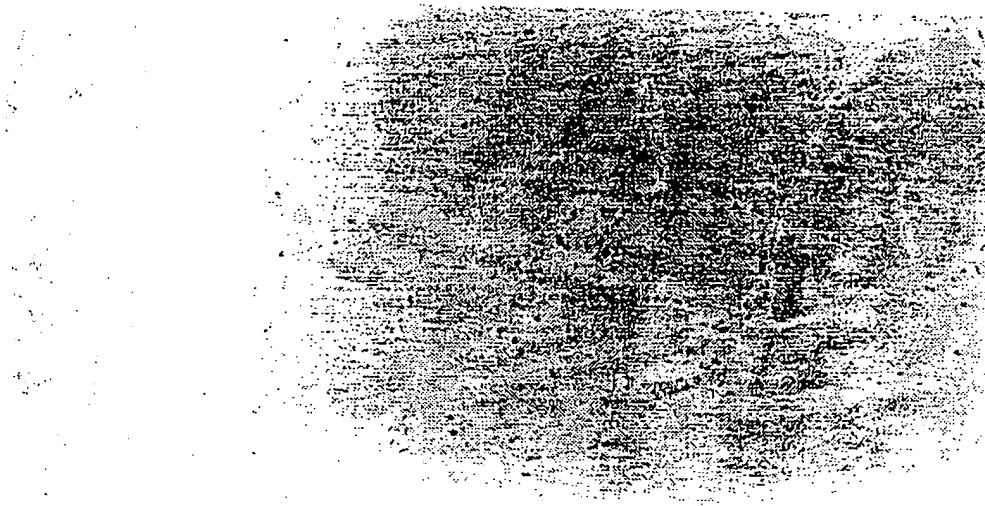


Fig. 9

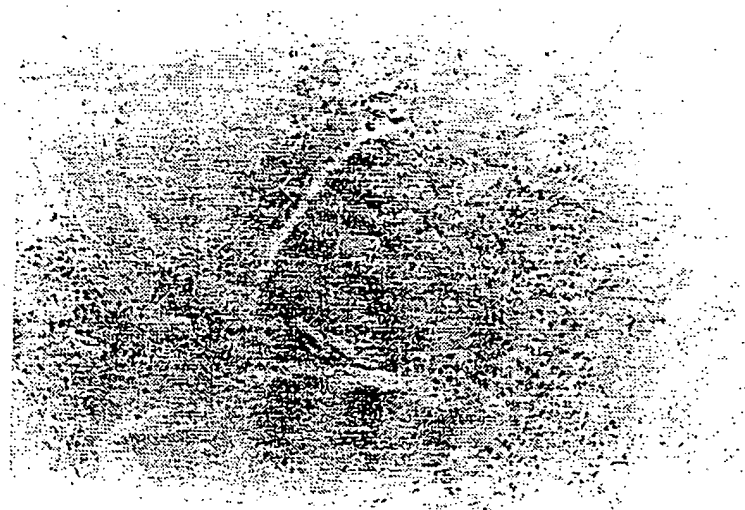


Fig. 10



Fig. 11

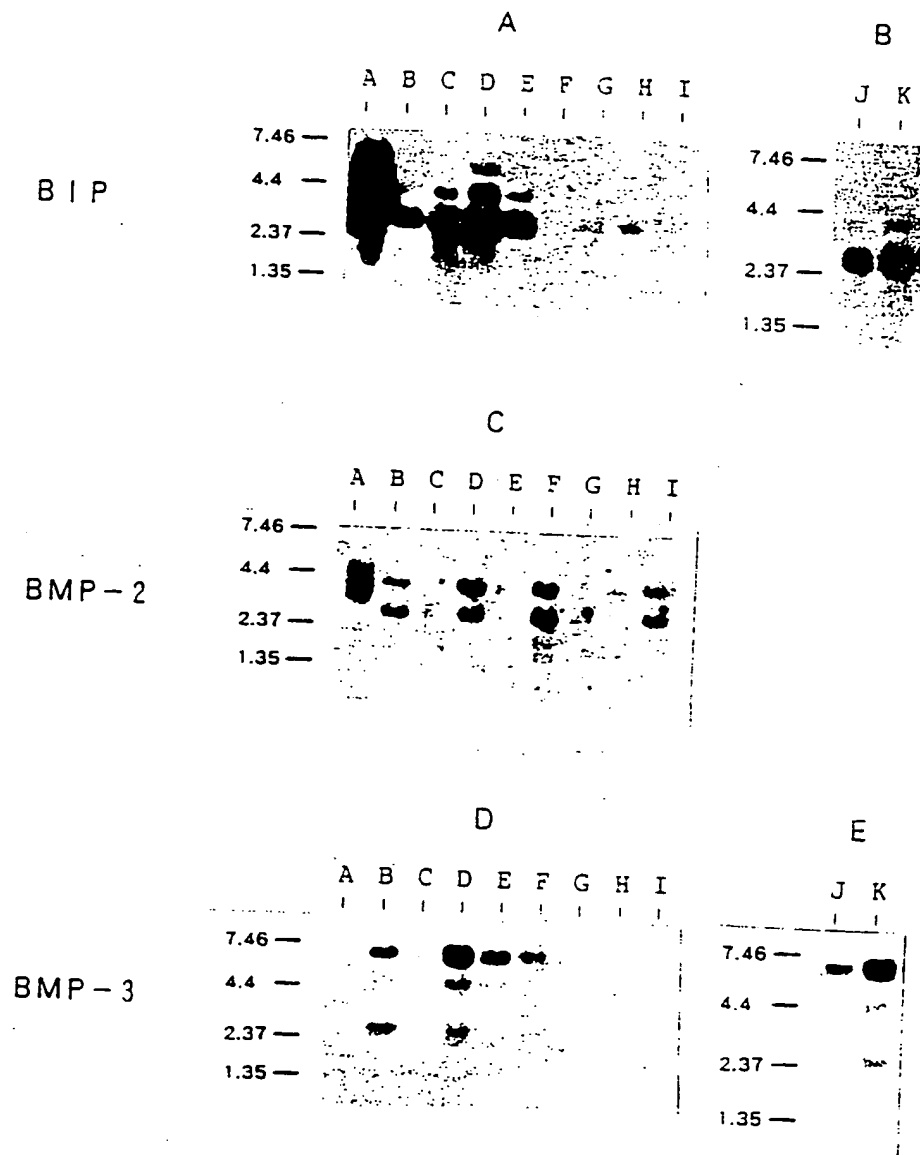


Fig. 12

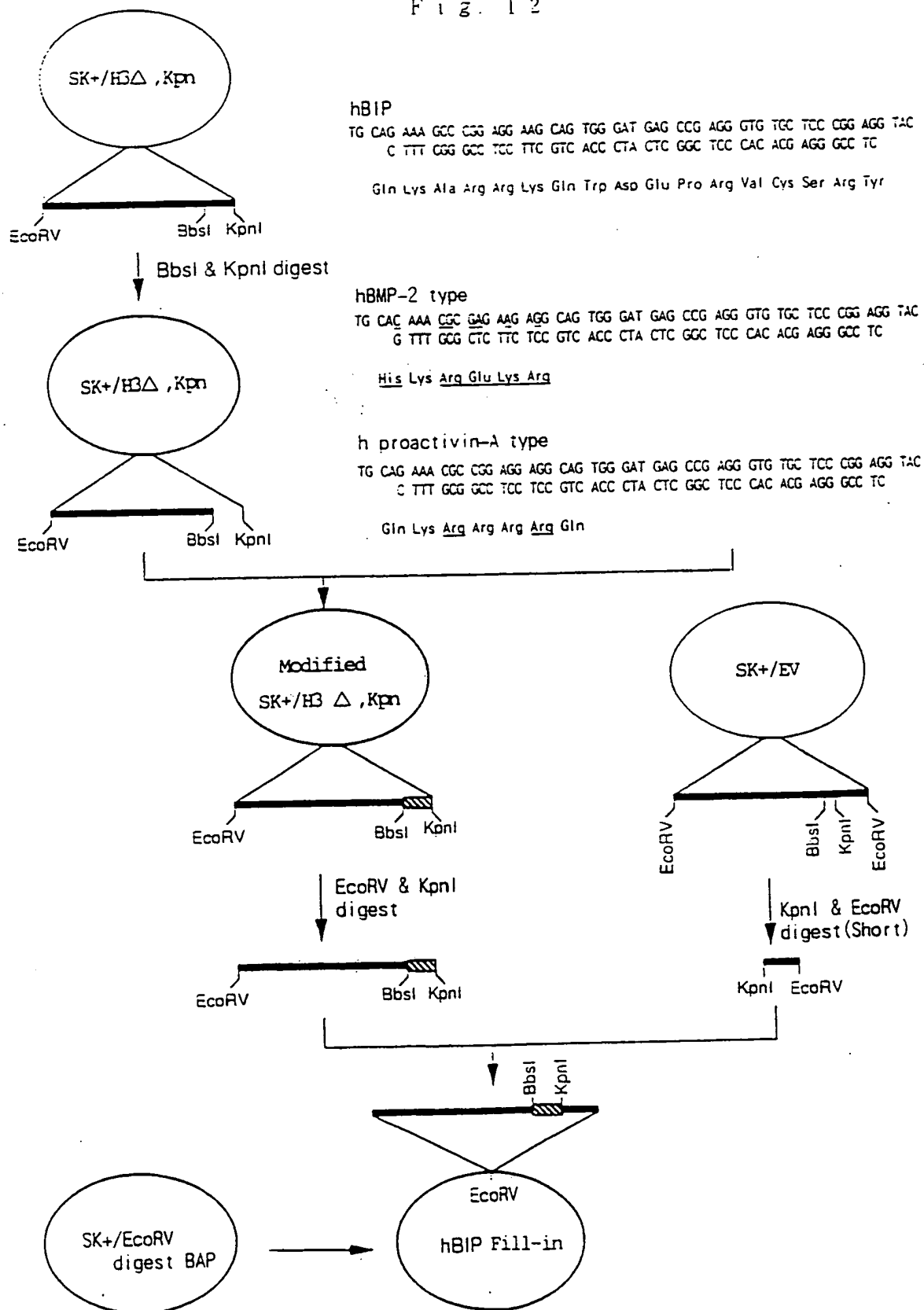
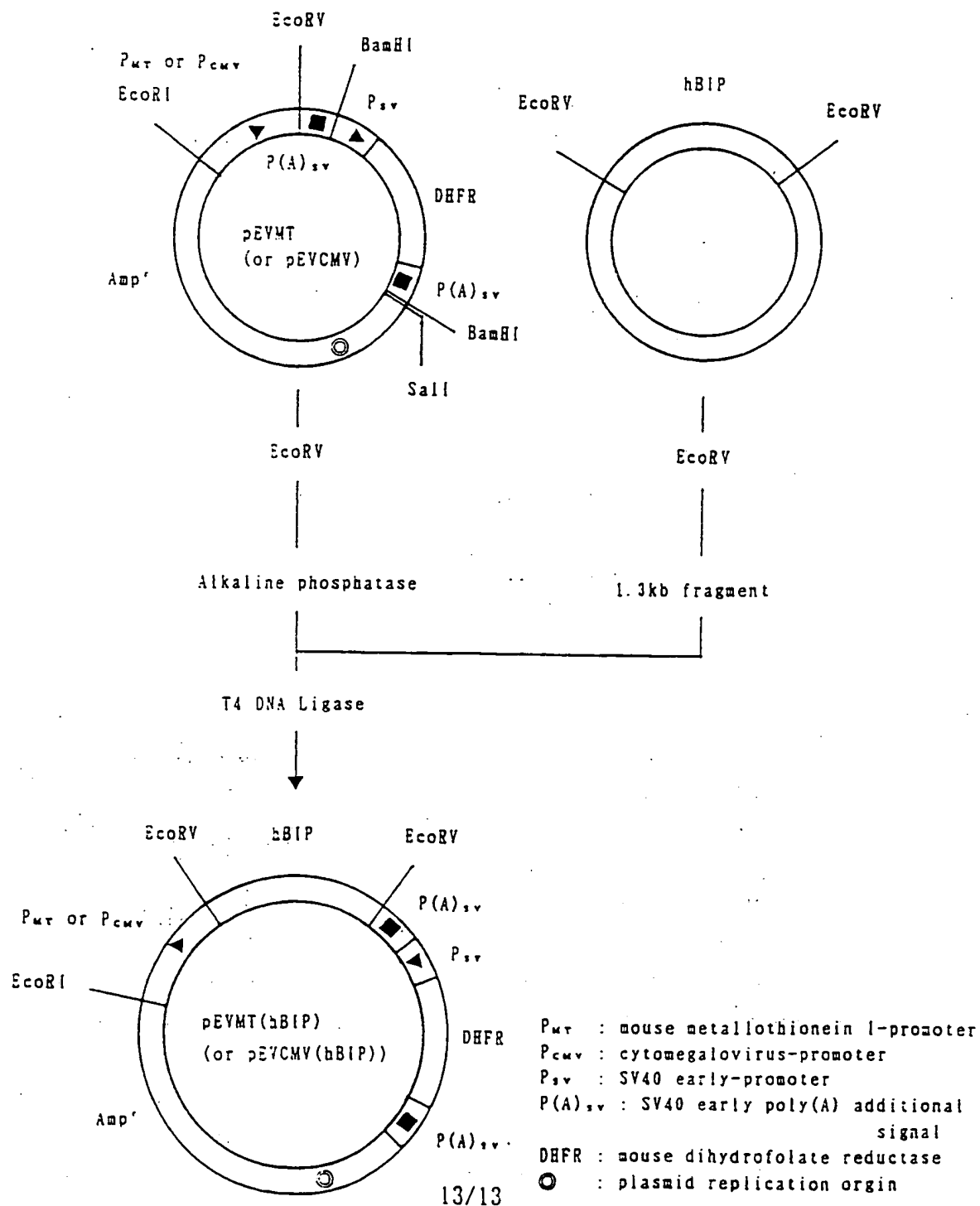


Fig. 13



## INTERNATIONAL SEARCH REPORT

International Application No

PCT/JP 93/00952

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 C12N15/12; A61K37/02; A61K9/22		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
Int.Cl. 5	C07K ; C12N ; A61K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
A	WO,A,8 800 205 (GENETICS INSTITUTE, INC.) 14 January 1988 see page 8, line 31 - page 13, line 1 see page 25 - page 28 see claims	1-11
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA vol. 87, no. 24, December 1990, WASHINGTON US pages 9843 - 9847 CELESTE, A. ET AL. 'Identification of transforming growth factor beta family members present in bone-inductive protein purified from bovine bone' see the whole document	1-11
<p><sup>10</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same parent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
25 OCTOBER 1993		05 -11- 1993
International Searching Authority		Signature of Authorized Officer
EUROPEAN PATENT OFFICE		ANDRES S.M.

Form PCT/ISA/210 (second sheet) (January 1985)